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Evaluation of antihyperglycemic activity of plants in northeast mexico

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Abstract: Diabetes mellitus is one of the most common non-contagious diseases. In 2017, The International Diabetes Federation reported that around 425 million people suffer from diabetes worldwide. Medications used for the treatment of diabetes lead to unwanted side effects, and thus, new safe drugs are necessary. Some natural plant-based products exhibit anti hyperglycemic activity and low toxicity. The aim of this study was to evaluate the antihyperglycemic activity (using both *in vitro* and *in vivo* models) as well as cytotoxicity of the extracts obtained from various plants. Nine extracts from a total of eight plant species were subjected to *in vitro* α-amylase and α-glucosidase inhibition assays. Subsequently, they were assessed through the *ex vivo* everted sac assay, and finally, the *in vivo* antihyperglycemic activity was evaluated. The extracts obtained from *Ceanothus coeruleus, Chrysactinia mexicana* and *Zanthoxylum fagara* inhibited the activities of α-amylase and α-glucosidase in the *in vitro* assays. Ethyl acetate and hydroalcoholic extracts from *Jatropha dioica*, hydroalcoholic extract from *Salvia ballotaeflora and Chrysactinia mexicana,* as well as methanolic extract from *Ricinus communis* and *Zanthoxylum fagara* significantly reduced the glucose uptake in the *ex vivo* everted intestinal sac test. All the eight extracts showed antihyperglycemic effect through the *in vivo* model of the Glucose Tolerance Test, using starch as the carbohydrate source. The antihyperglycemic effect of the extracts could be mediated through the inhibition of digestive enzymes and/or the absorption of glucose through the intestine. However, the mechanism of action for the hydroalcoholic extract of *Salvia texana* and the methanolic extract of *Turnera diffusa*, which showed a strong *in vivo* antihyperglycemic effect, is unclear.

Key words: α-amylase; α-glucosidase; Inverted sac; Antihyperglycemic activity *Zanthoxylum fagara.*

Introduction

Diabetes mellitus is a metabolic syndrome and is among the most common non-contagious diseases, along with cardiovascular diseases and cancer (1). The International Diabetes Federation (IDF) reported in 2019 that around 352 million people between the ages of 20 and 64 years worldwide suffer from diabetes (2). The disease is characterized by the inability to regulate the level of blood glucose, either due to insulin resistance in peripheral tissue and/or the inadequate quantity or quality of insulin secreted by beta cells of pancreatic islets (3).

There are various symptoms of diabetes mellitus, including polydipsia, polyuria, polyphagia and/or weight loss, numbness of hands and feet, etc. Uncontrolled diabetes mellitus for prolonged periods could lead to ketoacidosis, retinopathy, nephropathy, neuropathy, hypertension, stroke, etc. (4). To date, no effective treatment has been found for this disease. The current medications can only help control blood glucose levels within normal values for a limited time period per dose. The mechanisms of action of these medicines vary widely, although most of them act by the mechanisms related to insulin, either as analogues, stimulants for their production or absorption, or sensitizers. In addition, other drugs, such as carbohydrate digestion and absorption inhibitors and renal reabsorption inhibitors, have been described (5, 6). Almost all these medications have different side effects, together with the high cost of treatment. For this reason, it is necessary to find new products that could help control hyperglycemia with fewer side effects and lower cost.

The pathway for drug discovery includes preclinical trials involving animals; however, it often also includes *in vitro* assays before *in vivo* testing. The results obtained with *in vivo* models are widely accepted by both the pharmacy and the clinic, since such evaluation involves the entire organism and, therefore, its entire metabolism. However, animal testing is costly, consumes a lot of time, and requires the testing of a large number of products, especially a large number of animals (7).

Previously, several biologically active natural products have been studied and have been proved to show less toxic or side effects compared to some synthetic compounds (8). The ability of plant extracts to reduce blood glucose has been previously demonstrated using *in vitro*, *ex vivo*, as well *in vivo* models (6).

In vivo studies are essential to demonstrate the activity of any product; however, such studies require a large number of animals and/or samples to be analyzed, as well as a lot of time, resources, and effort. On the other

Table 1. Extracts of plants used.

	Plant name	Solvent Extraction	Voucher
1	*Ceanothuscoeruleus*	Water	024099
2	*Chrysactinia mexicana*	Methanol:Water (90:10)	024102
3	*Jatropha dioica*	Ethylacetate (EAc)	024077
4	*Jatropha dioica*	Methanol:Water (90:10) (MW)	024077
5	*Ricinuscommunis*	Methanol	006278
6	*Salvia ballotaeflora*	Methanol:Water (90:10)	015925
7	*Salvia texana*	Methanol:Water (90:10)	022298
8	*Turneradiffusa*	Methanol	023569
9	*Zanthoxylumfagara*	Methanol	015046

hand, high performance *in vitro* assays allows the evaluation of a large number of products within a short period of time. Potentially active products could then be selected for further evaluation through *in* vivo models (7).

Our workgroup has previously reported the optimization and validation of *in vitro* methods to evaluate the inhibitory activities of α-glucosidase (9) and α-amylase (10). The optimization and validation of these methods have been carried out in order to confirm the reliability of the results and select a reasonable number of extracts that could be assessed using the *in vivo* model. Products that exhibit an adequate level of activity in an *in vitro* model are more likely to succeed in the *in vivo* model, although *in vivo* systems are much more complex.

To evaluate the probability of success, we proposed to perform both *in vitro* and *in vivo* evaluation of extracts obtained from diverse plant species (available in our lab), regardless of their traditional use (not knowing whether or not they have biological activity, to include both active and inactive extracts). In this paper, we use two indicator assays of antihyperglycemic activity: 1) *in vitro* inhibitory assays of carbohydrate-related enzymes, such as α-amylase and α-glucosidase, and 2) glucose transporter inhibition assays from intestinal lumen to circulation. Subsequently, we evaluated the effect of the same products on postprandial hyperglycemia in an *in vivo* model through the Oral Starch Tolerance Test, using normoglycemic rats. We also evaluated the cytotoxicity of the extracts.

Materials and Methods

Reagents

The following reagents were purchased from Sigma-Aldrich (USA): α-glucosidase from *Saccharomyces cerevisiae* (12.4 U/mg); starch; sodium carbonate; monobasic potassium phosphate; p-nitrophenyl-alpha-D-glucopyranoside (p-NPG); potassium persulfate; sodium potassium tartrate tetrahydrate; porcine pancreas α-amylase (15 U/mg); acarbose; 3,4-Dinitrosalicylic acid (DNSA); Bovine Serum Albumin (BSA, Fraction V, 96-99% Albumin); sodium chloride (for cell culture); dibasic sodium phosphate; Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM); fetal bovine serum (FBS); doxorubicin; 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT); and dimethylsulfoxide (DMSO). Dibasic potassium phosphate and monobasic sodium phosphate were purchased from Fisher Scientific, USA. Sodium hydroxide was purchased from CTR, Mexico. Empagliflozin was purchased from AKos Consulting Solutions Deutschland GmbH. AccuChekPerforma glucometer was purchased from Roche.

Plants

All the extracts used in this study had been used in previous investigations by our working group. In general, the plant material was dried at room temperature and pulverized and subjected to extraction using different solvents as shown in Table 1. The extracts were then filtered and evaporated to dryness *in vacuo* and kept at a temperature of 4°C until further use.

α-Glucosidase inhibitory assay

For this assay, we used the already optimized and validated protocol described previously by Granados (9): 33 µL of the extract (final concentration between 20.6 to 660 µg/mL in 100 mM phosphate buffer, pH 6.8) and 17 µL of p-NPG (final concentration of 33.6 µg/mL) were mixed and incubated for 5 min at 37 °C. Subsequently, 17 µL of α-glucosidase enzyme (final concentration: 46.75 mU/mL) was added, and the mixture was incubated for 17.5 min at 37°C. Finally, 133 µL of sodium carbonate (final concentration: 66.5 mM) was added. Absorbance was measured at 405 nm using a plate reader. The absorbance value of the blank was subtracted from the absorbance value of the samples. A negative control (without inhibitor), with 100% activity, was used, and acarbose was used as a positive control. All experiments were performed in triplicates. The percent inhibition was calculated using Equation 1. The values of percent inhibition were plotted against concentrations. The concentration that inhibited 50% enzyme activity (IC_{50}) was interpolated from the curve.

Eq. 1: % inhibition = (100) $[(A_{\text{negative control}}-A_{\text{test}})/A_{\text{negative control}}]$

where, $A_{\text{negative control}}$ = Absorbance of the negative control (reaction mixture without adding the substance with known inhibitory activity). A_{test} = Absorbance of the test agent (positive control or agent to be evaluated).

α-amylase inhibitory assay

For this assay, we used the already optimized and validated method described previously by Granados (10). In a tube were mixed 100 µL One hundred microliters of different extracts (final concentration: 6 to 200 µg/mL in 100 mM phosphate buffer, pH 6.9) were mixed with 100 µL of α-amylase (final concentration of 0.15 U/mL in phosphate buffers) in separate tubes. This mixture was incubated for 7.2 min at 37 °C. Two

hundred microliters of DNSA (final concentration: 19.2 mM) was added and placed in a boiling water bath for 15.6 min. The mixture was then cooled and 100 µL of the mixture was transferred to a 96-well plate and diluted with 100 µL of water. The absorbance of the diluted mixture was measured at 540 nm using a Multiskan FC plate reader (Thermo Scientific). A sample blank (without enzyme) was included with each experiment. The absorbance value of the sample blank was subtracted from the absorbance value of the samples. A negative control (without inhibitor), with 100% activity, was used and acarbose was used as a positive control. All the experiments were performed in triplicates. The IC_{50} was determined as indicated in the previous section.

Cytotoxicity

Vero cells were maintained in DMEM medium containing 10% SFB, 1% L-glutamine, and 1% antibiotics, at 37°C under5% CO_2. Upon reaching 90% confluence, the cells were detached using trypsin, and after centrifuging at 3,500 rpm for 5 min, counted in a Neubauer chamber. Around 50,000 cells were seeded in each well of a 96-well microplate and incubated overnight under the same conditions (11). The medium was subsequently discarded and 200 µL of either blank medium (negative control) or medium containing either doxorubicin (positive control) or extracts (final concentrations between 0.5 - 500 µg/mL) was added to each well. Plates were incubated under same conditions for 48 h. Cell growth was checked under the light microscope to rule out contamination and the plaque content was decanted. Cell growth was revealed by adding 200 µL of 0.5 mg/mL MTT solution to each well and incubated for 3 h under standard conditions. The supernatant was decanted, and the crystals were dissolved in 200 µL of DMSO. After stirring the plates, the absorbance of each well was measured at 540 nm. All experiments were performed in quintuplicates on three different plates. The absorbance of each well was compared against the absorbance of the negative control (100% viability or 0% inhibition) and the percentage of cytotoxicity was calculated. Data from each plate were handled separately. Cytotoxicity percentages were plotted based on the extract or doxorubicin concentrations and the concentration corresponding to 50% cytotoxicity (CC_{50}) was determined by interpolation.

Intestinal absorption of glucose –*ex vivo* assay

For this assay, we used the protocol previously described by Nuñez et al. (12). Male Wistar rats (400 g each) were used and provided with water and food *ad libitum*. All animals were treated according to the guidelines of NOM-062-ZOO-1999 (13). The animals were sacrificed by cervical dislocation. An abdominal incision was made, and the small intestine was removed. Pieces of intestine (approximately 5 cm long) were isolated from the duodenum and the first part of the jejunum. Each piece was washed with saline solution and one end was ligated using the surgical thread. With a glass rod, the ligature was moved to the opposite side. Once the piece of intestine was reversed, the tip of a Pasteur pipette was introduced and attached to the free end. The intestinal sac was filled with physiological solution (0.15 M NaCl, pH 7.4) to verify the absence of any leak.

In order to obtain the baseline absorption data, one of the intestinal sacs was incubated in saline at 37°C with constant bubbling for 3 h (negative control). Other intestinal sacs were incubated in either saline solution containing empagliflozin (5 mg/dL, positive control) other extract (5, 10, and 20 mg/dL), under same conditions. During the incubation, the solution inside the intestinal sac was aliquoted at different times (0, 30, 60, 90, 120, 150, and 180 min). The concentration of glucose absorbed through the intestine was measured with an AccuChek Performa portable glucometer (Roche). A graph of glucose absorption kinetics was obtained. The area under the curve (AUC) was calculated using the Riemann method (sum of midpoints), to determine the effect throughout the kinetics. In addition, the percentage of absorption was determined at each time point and the significant difference, with respect to the negative control, was established at 90 min.

Oral starch tolerance test –*In vivo* model

Male, normoglycemic Wistar rats with an average weight of 220 g were used. All animals were kept in the bioterium with temperatures between 20 and 25 °C, relative humidity between 40%and 50%, and 12-h light-dark cycle, with water and food provided ad libitum. All animals were treated under the guidelines of NOM-062-ZOO-1999. We used the procedure previously reported by Yusoff et al. (14) with slight modifications. Briefly, the rats were divided into groups of five rats:

Group 1: Water (1 mL/kg).
Group 2: Corn starch (1 g/kg).
Group 3: Corn starch plus acarbose (0.5 mg/kg).
Group 4: Starch plus a protein hydrolyzate of the legume *Mucuna pruriens* (PPHT: 0.5 mg/kg), which was included as a control extract and was included for a comparative purpose (12).

All the treatments were orally administered. Different concentrations of each of the 8 extracts were evaluated (0.5, 2.5, and 5 mg/kg).

After a 5-h fast, basal glucose level was recorded in whole blood (extracted from the tip of the tail) with the help of a portable AccuChek Performa glucometer (Roche). Subsequently, the carbohydrate load (corn starch: 1 g/kg) was administered orally with or without inhibitor (acarbose, PPHT, or extract), and blood glucose level was measured at 15, 30, 45, 60, and 120 minutes. Normalized blood glucose curves (ratio of glucose concentration at each time and zero time for each rat) were constructed for each group and the AUC was calculated using the Riemann method to determine the inhibitory effect. In addition, the percentage reduction of the glucose peak was determined at 30 min, considering the peak generated by starch as 100% elevation.

Statistical analysis

In vitro and *ex vivo* experiments were performed in triplicates, while *in vivo* experiments were done in quintuplicates. Data were expressed as mean ± standard deviation. The results of each extract or positive control were compared with a negative control, using one-way analysis of variance (ANOVA) followed by Dunnett´s test. A value of $p < 0.05$ indicated statistical significance.

Table 2. Inhibition of digestive enzymes.

	Plant extract	α-glucosidase	α-amylase
		CI_{50} (µg/mL)	CI_{50} (µg/mL)
1	*Ceanothuscoeruleus*	17.7 ± 1.7	14.0 ± 0.9
2	*Chrysactinia mexicana*	> 660	9.09 ± 0.6
3	*Jatropha dioica- AcE*	141.9 ± 6.4	> 50
4	*Jatropha dioica MW*	> 660	> 50
5	*Ricinuscommunis*	> 660	> 50
6	*Salvia ballotaeflora*	> 660	> 50
7	*Salvia texana*	> 660	> 50
8	*Turneradiffusa*	> 660	> 50
9	*Zanthoxylumfagara*	107.9 ± 2.0	4.8 ± 0.1
	Acarbose	164.3 ± 1.7	2.9 ± 0.1

Results

No extract showed cytotoxicity at the maximum concentration used (500 µg/mL), except the ethyl acetate extract of *J. dioica*, which showed a CC_{50} of 257 µg/mL, while doxorubicin displayed a CC_{50} of 12.8 ± 0.5 µg/mL.

The inhibitory effect of the extracts on the activity of the enzymes involved in the digestion of carbohydrates is shown in Table 2. Extracts from *C. coeruleus* and *Z. fagara* inhibited the activities of both α-amylase and α-glucosidase, while the hydroalcoholic extract of C. Mexicana inhibited the activity of α-amylase and the ethyl acetate extract of *J. dioica* inhibited the activity of α-glucosidase.

Glucose absorption was evaluated through the *ex vivo* test using the inverted intestinal sac. Glucose absorption kinetics were first assessed without any inhibition, that is, with a negative control (Figure 1). The graph of the negative controls exhibited a correlation coefficient of 0.9943; the approximate flow of glucose absorption through the intestinal wall in this group was 50 mg/dL every hour. The kinetics in the presence of 5 mg/dL of empagliflozin, a sodium-glucose linked transporter (SGLT) inhibitor, displayed an excellent correlation coefficient of 0.9932, with an absorption flow of around 33 mg/dL every hour (Figure 1). To determine the reduction of glucose absorption by the positive control, the area under the curve (AUC) was calculated. The AUC of the negative control was considered to be 100% and a 38.4% reduction in glucose uptake through the intestine was determined in the presence of empagliflozin.

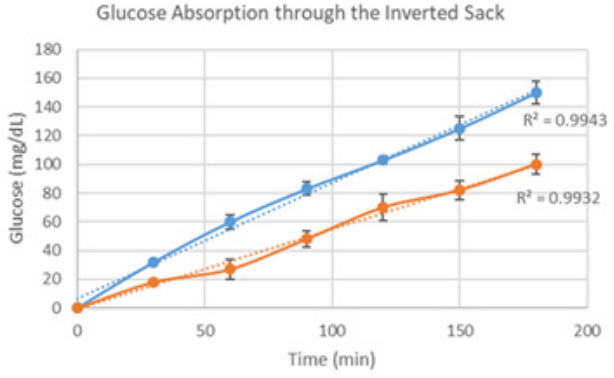

Figure 1. Glucose absorption kinetics. Blue: negative control. Orange: positive control.

Each extract was subjected to the absorption assay using the inverted sac, at three different concentrations, and respective absorption kinetics were obtained. The AUC of each experiment were determined and statistically compared with those obtained for the negative control. The ethyl acetate extract of *J. dioica* as well as the methanolic extracts of *S. ballotaeflora*, *R. communis*, and *Z. fagara* significantly reduced glucose absorption (Figure 2). In addition to the AUC, the percentage of glucose absorbed up to 90 minutes was also determined for each extract (Figure 3). At 90 min, *S. ballotaeflora* extract inhibited glucose absorption at three concentrations; the hydroalcoholic extract of *J. dioica* was active only at the two highest concentrations; interestingly, the level of reduction of glucose absorption by 20 mg/dL of the *C. mexicana* extract was comparable to that of the positive control. On the other hand, some extracts showed an inverse effect, that is, they led to a lower inhibition of glucose absorption than the negative control.

The results of the *in vitro* tests showed that extracts from five plants could potentially be used as antihyper-

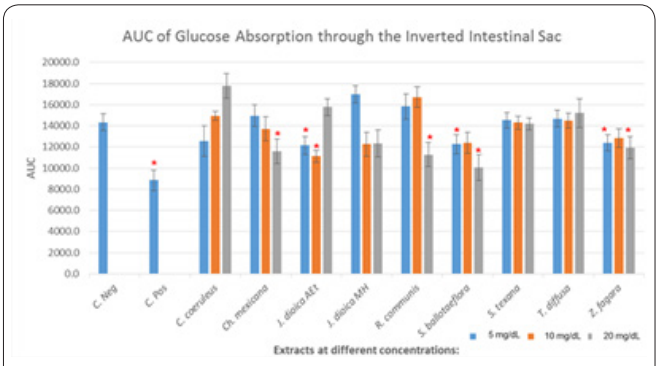

Figure 2. AUC of glucose absorption of controls and extracts (* p <0.05).

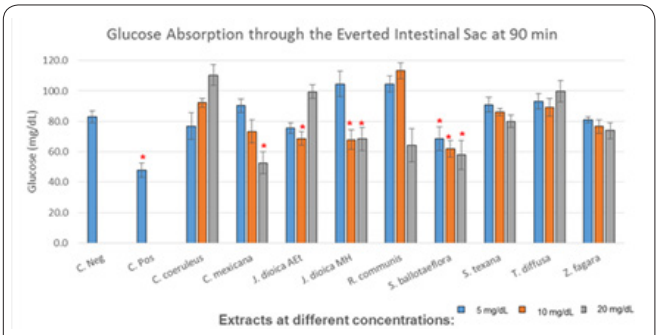

Figure 3. Glucose absorption at 90 min of exposure of controls and extracts. (* p <0.05).

glycemic agents: *C. coeruleus*, *Z. fagara*, *J. dioica* EAc, *J. dioica* MW, *R. communis*, and *S. ballotaeflora*. Thus, they were expected to produce positive results in the *in vivo* model. On the other hand, the *S. texana* and *T. diffusa* extracts showed no inhibitory activity in *in vitro* analysis.

All *in vivo* trials were performed following the guidelines of Good Practices for the Care and Management of Experimental Animals. First, the kinetics of Oral Starch Tolerance was established (Figure 4, blue line). The hyperglycemic peak was obtained 30 min after the administration of starch; subsequently, the blood glucose levels return to the initial values. When starch was administered along with acarbose (positive control), the hyperglycemic peak (obtained after 30 min) decreased significantly by around 65.7%.

In order to evaluate the effect of acarbose on the kinetics, the reduction in the degree of hyperglycemia was determined by calculating the AUC, considering the AUC of the starch as 100%. Acarbose administration was observed to produce a reduction of approximately 29.3% in the AUC values.

On the other hand, the behavior of the control extract (PPH) was comparable to that of acarbose, with a reduction of around 65.7% in the hyperglycemic peak at 30 minutes and around 33.4% in the AUC value compared to the kinetics of starch.

All extracts were evaluated under the same conditions at three different doses (Figure 5). In all cases, a significant decrease in the AUC with respect to starch control was observed, with the exception of the hydroalcoholic extract of *J. dioica* at the lowest dose. Interestingly, three extracts were more active than the acarbose (positive control), including the extracts of *S. ballotaeflora*, *J. dioica* (EAc), and *T. diffusa.*

Normalized glucose in the hyperglycemic peak (30 min) for each concentration of each extract is shown in Figure 6. Four extracts displayed a significant reduction in the hyperglycemic peak at 30 minutes, while four extracts did not show reduction in the hyperglycemic peak.

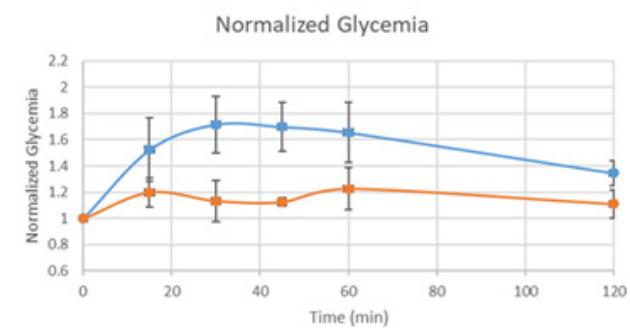

Figure 4. Normalized glucose kinetics. Blue: negative control. Orange: positive control.

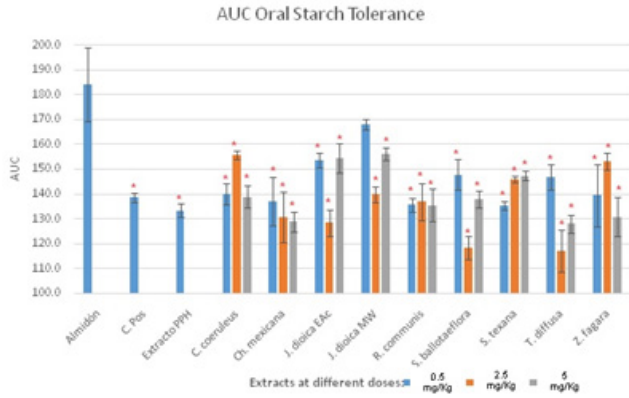

Figure 5. AUC oral tolerance to starch controls and extracts. (*p <0.05).

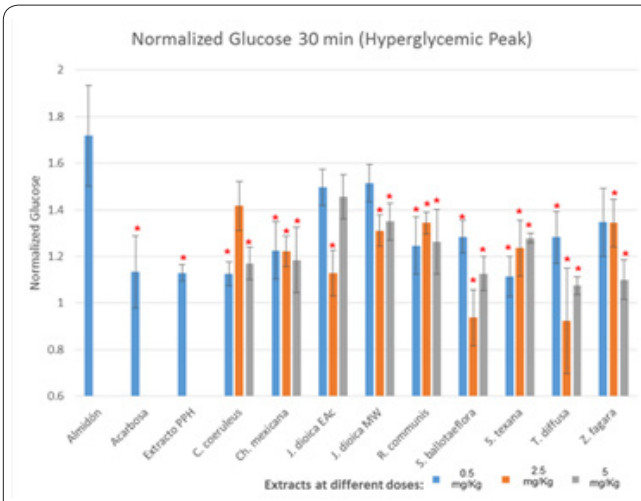

Figure 6. Normalized glucose at 30 min of exposure to controls or extracts. (*p <0.05).

Discussion

All the plants used for this study were collected from Nuevo León and were readily available in our laboratory. To the best of our knowledge, this was the first study that demonstrated the antihyperglycemic activities of the aqueous extract of *C. coeruleus*, methanolic extract of *Z. fagara*, ethyl acetate extract of *J. dioica*, and hydroalcoholic extracts of *J. dioica*, *S. ballotaeflora*, and *S. texana*.

Several species of *Ceanothus* genus have previously been reported to exhibit antimicrobial activities (15,16). In the present work, the aqueous extract of *C. coeruleus* strongly inhibited the activities of α-amylase and α-glucosidase. Inhibition of such enzymes could lead to a decrease in glucose absorption, and in turn, blood glucose levels. The extract of *C. coeruleus* showed no effect on the glucose uptake in *in vitro* analysis, but showed anti-hyperglycemic activity in the *in vivo* model. Our results suggested that it acts via the inhibition of digestive enzymes.

The methanolic extract of *Z. fagara* markedly inhibited the activities of both α-amylase and α-glucosidase. It significantly decreased the intestinal glucose absorption, although this inhibitory effect was significant at longer intervals, over 90 min. In this study, the inhibitory effect of the methanolic extract of *Z. fagara* on glucose absorption may explain its antihyperglycemic effect in *in vivo*. To date, for *Z. fagara*, only larvicidal activity has been reported (17); however, several species of the genus *Zanthoxylum* have been reported as inhibitors of digestive enzymes (18,19) and as antidiabetics (20).

Silva et al. (21) reported the antiviral activity of hydroalcoholic extract of *J. dioica*, while Araujo et al. (22) showed that the aqueous extract of *J. dioica* is neither cytotoxic nor genotoxic. The ethyl acetate extract showed moderate cytotoxicity on the Vero cells. In ad-

dition, it inhibited α-glucosidase activity and intestinal glucose absorption at significantly lower concentrations than acarbose. It exhibited an antihyperglycemic effect only at the dose of 2.5 mg/kg; however, the hyperglycemic peak at 30 min was lower than that obtained for positive control. Our results suggested that it acts via both the inhibition of α-glucosidase and the reduction of intestinal absorption. On the other hand, the hydroalcoholic extract of *J. dioica* did not exhibit enzymatic inhibition, only mildly affected the intestinal absorption of glucose (only at higher concentrations), and showed moderate antihyperglycemic activity at high doses. Our results suggested that it acts via the reduction of intestinal glucose absorption.

Recent studies have demonstrated the antidiabetic activity of the aqueous and hydroalcoholic extracts of *R. communis* leaves as well as the cytotoxicity and genotoxicity of its methanolic extracts at high concentrations (23,24). In the present work, the methanolic extract of *R. communis* did not inhibit the digestive enzymes and affected the glucose absorption only at the concentration of 20 µg/mL. However, the extract showed a significant antihyperglycemic effect. It is hypothesized that the antihyperglycemic activity of this extract is associated with its antidiabetic activity reported by Gad (23). Though, its mechanism of action is still unclear, it is hypothesized that it might act via the reduction of intestinal glucose absorption.

Some species of the genus *Salvia* have been reported to show antidiabetic activity (25,26). The hydroalcoholic extract of *S. ballotaeflora* did not show any inhibitory activity for any of the two digestive enzymes; however, it inhibited intestinal glucose absorption. It also exhibited marked antihyperglycemic effect greater than the positive control. These findings suggested that its antihyperglycemic activity is mediated via the reduction of intestinal absorption. On the other hand, the hydroalcoholic extract of *S. texana* showed no inhibition of enzymatic activity and intestinal absorption; however, in *in vivo* model, it moderately affected the kinetics of hyperglycemia and the hyperglycemic peak.

Finally, both the hydroalcoholic extract of *S. texana* and the methanol extract of *Turnera diffusa* showed no activity through the *in vitro* assays that we used. However, methanol extract of *Turnera diffusa* exhibited the most prominent *in vivo* antihyperglycemic activity, comparable to that of acarbose. Recently, Parra et al. (27) reported the isolation of a terpenoid compound from this plant species, which is responsible for its antidiabetic activity. The mechanism through which this extract exerts its antihyperglycemic and/or antidiabetic activity is still unknown.

The results obtained with most of the extracts exhibited the usefulness of conducting *in vitro* assays prior to using the *in vivo* models. In addition, *in vitro* assays could also help in elucidating the mechanism of action of any biological agent. However, the results obtained for *T. diffusa* demonstrate that sometimes this strategy could lead to misrecognition of biologically active extracts.

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Conflict of Interest

No conflict of interest has been declared by the authors.

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